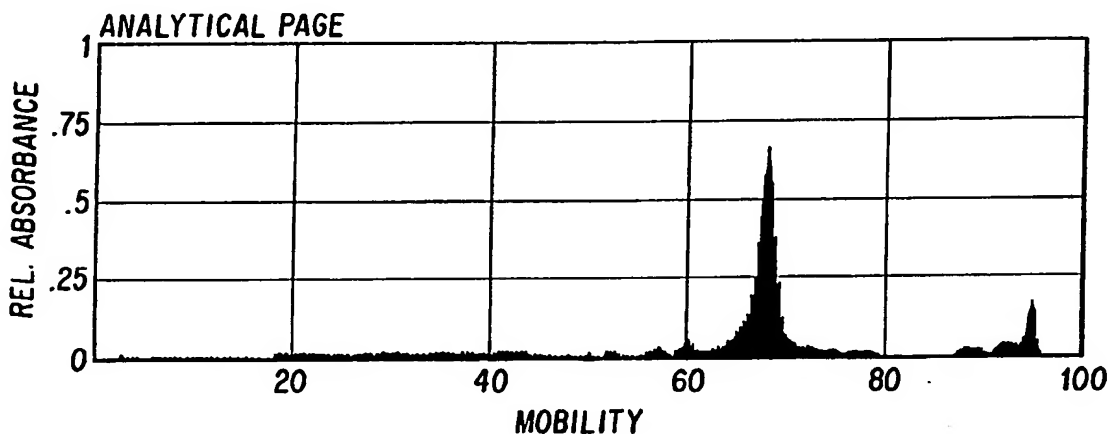




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12P 21/00, C07K 1/14, 3/02 C07K 3/12, 3/28	A1	(11) International Publication Number: WO 90/00200 (43) International Publication Date: 11 January 1990 (11.01.90)
(21) International Application Number: PCT/US89/01917 (22) International Filing Date: 5 May 1989 (05.05.89) (30) Priority data: 211,904 27 June 1988 (27.06.88) US (71) Applicant: GENEX CORPORATION [US/US]; 16020 Industrial Drive, Gaithersburg, MD 20877 (US). (72) Inventors: FINKELMAN, Malcolm, A., J. ; 410 North, 5225 Pooks Hill Road, Bethesda, MD 20814 (US). LEE, Timothy, Kwok-Tim ; 9701 Fields Road, Apartment 406, Gaithersburg, MD 20878 (US). (74) Agents: GOLDSTEIN, Jorge, A. et al.; Saidman, Sterne, Kessler & Goldstein, 1225 Connecticut, N.W., Suite 300, Washington, DC 20036 (US).		(81) Designated States: AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FI, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>

(54) Title: THERMAL RELEASE OF RECOMBINANT PROTEIN INTO CULTURE MEDIA

**(57) Abstract**

The invention relates to a method for the isolation of a substantially pure polypeptide from a recombinant bacterial host by culturing the host under recombinant protein-producing conditions followed by heating the aqueous nutrient medium to 50-100°C for a time not to exceed 1 hour and recovering the substantially pure polypeptide so produced.

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-1-

TITLE OF THE INVENTION

Thermal release of recombinant protein into culture media.

FIELD OF THE INVENTION

The invention relates to methods for the isolation of substantially pure polypeptides from recombinant micro-organisms.

BACKGROUND OF THE INVENTION

The use of bacteria to express foreign genes has faced many practical and biological problems, including the instability of the polypeptide due to proteolysis, low levels of expression, and precipitation of the protein product. Precipitation is correlated to misfolding and lack of biological activity of the protein after purification. To solve these problems, a variety of techniques have been developed to enhance the expression of gene products by transformed hosts. These methods include the use of various promoters to allow the regulation of the level of expression, gene fusions to stabilize normally unstable proteins in the cell, and the use of signal peptides to translocate proteins out of the cytoplasm to the periplasmic space. For example, Abrahamsen, L., et al., European Patent Application Publication No. 0 225 860,

published June 16, 1987, discloses methods for the isolation of expressed gene products having signal sequences from E. coli by induction of filamentous growth, where the expression of the desired gene product is dependent on the heat shock response. This response results in a quantitative leakage of periplasmic located proteins to the growth medium at temperatures of 30-42°C. Using these conditions, high expression and export of protein A was reported. However, this method is useful only for genes encoding signal sequences fused to protein A. Therefore, it is not widely applicable to all genes.

Proteins produced by host cells are normally trapped within the cells or secreted into the surrounding growth medium. In the former case, the cells must be ruptured to permit the desired protein to be isolated, whereas in the latter case, it can be separated from the growth media. Even in the case of secreted proteins, the preparation from which the protein is to be isolated is relatively complex, containing a variety of other substances. Despite efficient separation techniques, both the purity and the yield of the desired protein may be low. Lofdahl, S., et al., PCT application, publication no. WO 84/03103, published 16 August 1984.

Lofdahl et al., supra, have developed methods for selectively isolating a desired protein or polypeptide by constructing a recombinant vector containing a DNA sequence coding for the desired protein or polypeptide which is operatively linked to a DNA sequence coding for protein A. The expressed fusion protein is then selectively isolated by absorbing onto an IgG-supporting carrier, which binds protein A, followed by desorption of the fusion protein. The fusion protein is then cleaved at a unique cleavage site with a cleavage agent, which may include proteases, hydroxylamine, cyanogen bromide or formic acid, to give the purified protein.

-3-

Despite the above-described methods for isolating polypeptides produced by recombinant means, a need continues to exist for methods which allow the selective production of substantially pure polypeptides.

SUMMARY OF THE INVENTION

The invention relates to a method for the isolation of a substantially pure polypeptide expressed by a recombinant host comprising:

(a) cultivating on an aqueous nutrient medium, under recombinant protein producing conditions, a micro-organism transformed by a vector comprising a DNA sequence encoding said polypeptide, said vector further comprising expression signals which are recognized by said host and which direct the expression of said DNA sequence;

(b) heating said aqueous nutrient medium to 50-100°C for a time not to exceed one hour to cause release of substantially pure polypeptide from the host; and

(c) recovering said substantially pure polypeptide from the aqueous nutrient medium.

Unexpectedly, it has been discovered that when a recombinant host is heated to 50-100°C for a time not to exceed 1 hour, high levels of substantially pure polypeptide are released into the growth media. This method does not rely on filamentous growth of the host as taught by Abrahamsen et al., supra, and in fact results in almost immediate and complete cell death at 70-80°C.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a densitometric scan of a whole cell E.

coli extract of protein G separated by polyacrylamide electrophoresis.

Figure 2 depicts a densitometric scan of the supernatant separated by polyacrylamide electrophoresis after heating an aqueous nutrient medium containing E. coli transformed with a vector containing a gene encoding protein G to 80°C for 5 minutes. The major peak is protein G.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention is directed toward a method for the isolation of substantially pure polypeptide from a recombinant bacterial host by culturing the host under protein-producing conditions, heating the aqueous nutrient medium to 50-100°C for a time not to exceed 1 hour, and isolating the substantially pure polypeptide so produced. Although longer heating times and higher temperatures may be utilized, they are not preferred since degradation of the protein occurs under these harsher conditions.

Preferred bacterial hosts include Gram-negative organisms, in particular, E. coli, Erwinia sp. and Klebsiella sp. The most preferred host is E. coli.

Preferably, the aqueous nutrient medium is heated to 50-100°C for about 5 minutes. Most preferably, the aqueous nutrient medium is heated to about 80°C for about 5 minutes. Under the most preferable conditions, substantially pure polypeptide is released into the culture media free from significant degradation. The method has the added advantage that endogenous proteases are inactivated by the high temperatures of the method, thus preventing proteolysis of the polypeptide.

By the term "polypeptide" is intended protein G or protein G variants having the immunoglobulin binding proper-

-5-

ties of protein G, protein A or protein A variants having the immunoglobulin binding properties of protein A, and those polypeptides which may be produced in substantially pure form by the method of the invention, which include any peptide which does not irreversibly denature at 50-100°C and which can penetrate the cell wall during the heating procedure. Such proteins may include, but are not limited to, small proteins such as hormones, e.g., parathyroid hormone, growth hormone, gonadotropins (FSH, luteinizing hormone, chorionogonadotropin), insulin, ACTH, prolactin, placental lactogen, melanocyte stimulating hormone, thyrotropin, calcitonin, enkephalin, angiotensin, and small cytokines.

In a preferred embodiment, the recombinant microorganism contains a vector containing the gene which encodes protein G. Vectors which contain the genes which encode protein G and protein G variants which have the immunoglobulin binding properties of protein G are described, for example, in International Application PCT/US87/00329, co-pending U.S. Application Serial No. 063,959, filed June 19, 1987, and co-pending U.S. Application Serial No. 209,236, filed June 20, 1988, the disclosures of which are incorporated by reference herein in their entirety. The vectors may incorporate promoters derived from, for example, bacteriophage, especially bacteriophage lambda, the E. coli tryptophan operon, the E. coli lac operon, the E. coli β -glucuronidase locus, etc. Suitable recombinant hosts, disclosed in copending U.S. Application Serial No. 063,959, include E. coli GX7820, E. coli GX7823, E. coli GX8464, and E. coli GX8465. Most preferably, the production organism is the transformed host E. coli GX1201 or E. coli GX6705, which contains the vector pGX5204 or a degenerate variant thereof.

By the method of this invention, high levels of substantially pure polypeptides can be obtained under conditions

where they may be easily isolated from the fermentation broth. By the term "substantially pure" is intended polypeptides which are substantially one major band by SDS-PAGE polyacrylamide electrophoresis and which contain only minor amounts of other proteins which normally contaminate a whole cell lysate, as evidenced by the presence of other minor bands.

The recombinant cells may be cultivated under any physiologically compatible conditions of pH and temperature, in any suitable nutrient medium containing assimilable sources of carbon, nitrogen and essential minerals that support cell growth. Recombinant protein-producing cultivation conditions will vary according to the type of vector used to transform the host cells. For example, certain expression vectors comprise regulatory regions which require cell growth at certain temperatures, or addition of certain chemicals to the cell growth medium, to initiate the gene expression which results in the production of the recombinant polypeptide. Thus, the term recombinant "protein-producing conditions," as used herein, is not meant to be limited to any one set of cultivation conditions.

The expressed protein may be recovered from the fermentation broth using any of the methods commonly known to those skilled in the art. As a first step, dead cells and insoluble debris are removed from the aqueous nutrient medium by filtration or centrifugation. Protein G or other recombinant protein may then be purified from the fermentation media using standard procedures such as absorption to immobilized immunoglobulin, as described by Sjoquist, U.S. Patent No. 3,850,798 (1974), ion exchange or gel chromatography, precipitation (e.g., with ammonium sulfate), dialysis, filtration or a combination of these methods.

The method of the invention is not limited to expression of polypeptides which have leader sequences. In addition, the

-7-

invention does not depend on induction of filamentous growth of E. coli as described by Abrahamsen et al., European Patent Application 0 225 860. Cell death is complete upon heating at 70-80°C; thus, induction of filamentous growth at these temperatures is not possible. Moreover, since the fermentation media is heated to 50-100°C in as little time as 5 minutes, there is little time for meaningful physiologically related morphological changes.

Although the inventors do not wish to be bound by any particular theory, it is hypothesized that the surprising level of purity of the expressed gene products, which results from heating at 50-100°C, is due to a dialysis-like effect. Such an effect might be expected if the heat treatment causes complete loss of membrane integrity. As the temperatures utilized in the practice of the invention likely cause the complete destruction of the cytoplasmic and outer cell membranes, the only remaining barrier to the release of the cell contents would be the peptidoglycan (PG) layer of the cell envelope. The protoplasmic contents are likely trapped within the PG barrier as a result of heat denaturation and coagulation. This theory is supported by the observation of condensed, granular cytoplasmic material by microscopic inspection after heat treatment. Soluble polypeptide, for example protein G, diffuses through the PG layer without significant impediment, by a dialysis-like mechanism. Thus, the polypeptides which may be recovered according to the invention, as listed above, are those which remain soluble and do not denature upon heating.

The following examples are provided to illustrate the invention, and are not to be construed as limiting the invention in any manner.

EXAMPLE 1

Preparation of Substantially Pure Protein G

Recombinant E. coli containing a vector which encodes a protein G variant having the immunoglobulin binding properties of protein G (GX8465, see U.S. Application Serial No. 063,959, filed June 19, 1987, incorporated by reference herein) was cultivated in an aqueous nutrient medium (see Table 1) at 32°C, 800 rpm, 1 vvm, pH 7.2 \pm 0.1 (titrants: 10% NaOH, 2M H₃PO₄). After cultivation at 32°C for 6-12 hours, expression of recombinant protein G was induced by raising the broth temperature to 42°C for 1 hr. After 1 hr at 42°C, the broth temperature was reduced to 39°C and maintained at that temperature for 3 hours. As shown in Figure 1, separation of a whole cell extract by SDS-PAGE electrophoresis followed by densitometric scanning of the gel shows that the extract is substantially contaminated by other proteins. The broth was then heated to 80°C and maintained at that temperature for one hour. Samples were removed periodically for analysis by gel electrophoresis (SDS-PAGE). Both cell pellets and cell free media were analyzed at 0, 15, 30 and 60 minutes after heating to 80°C.

Before heating, the pellet contained substantially impure protein G. At this time, no protein G was detected in the fermentation media. At time = 0 (when the temperature reached 80°C), a considerable band of substantially pure protein G was found in the cell-free medium. The corresponding cell pellet showed reduced levels of protein G. At time = 15, only a trace of protein G remained within the pellet. After 30 minutes, no protein G was detectable in the pellet. In contrast, after heating for 15 minutes at 80°C, substantially pure protein G was observed in the supernatant. The relative purity of the protein G in the supernatant did not substan-

-9-

tially decline after continued heating at 80°C for one hour. However, some chemical alteration, as evidenced by a fuzziness of the protein G band, was observed after 30 min. at 80°C.

Thus, heating at 80°C results in the release of substantially all of the protein G into the supernatant and retention of other proteins within the cells.

TABLE 1
Fermentation Media Composition

<u>Ingredients</u>	<u>Concentration</u>
Acid digest casein	30 g/l
* Glucose	30 g/l
K ₂ HPO ₄	5 g/l
(NH ₄) ₂ SO ₄	3 g/l
MgSO ₄ ·7H ₂ O	0.25 g/l
CaCl ₂ ·2H ₂ O	0.1 g/l
M44 salts (100 X)	10 ml/l
* Biotin	0.1 mg/l
* Nicotinamide	1.0 mg/l
* Ampicillin	100 mg/l
Distilled H ₂ O	dilute to 1360 ml
SAG 4130	0.25 ml/l

* = post autoclaving additions

EXAMPLE 2
Release of Protein G at 70°C

The experiment in Example 1 was repeated using a 70°C heat treatment instead of an 80°C heat treatment to induce release of protein G. At this temperature, the release of protein G into the broth does not appear to be complete.

-10-

Although substantially pure, the protein G appears to degrade after extended heating at 70°C as evidenced by fuzzy bands on the polyacrylamide electrophoresis gel.

EXAMPLE 3

Release of Protein G at 80°C for 5 Minutes

Example 1 was repeated, except that the holding period at 80°C was reduced to about 5 minutes. This brief exposure resulted in essentially quantitative release of substantially pure protein G as observed by SDS PAGE electrophoresis (Figure 2). In addition, the apparent degradation of protein G due to prolonged heating (as evidenced by the widening of the protein G band on the gel) was greatly reduced.

EXAMPLE 4

Preparation of Substantially Pure Protein A

Recombinant E. coli containing a vector which encodes Protein A, strain NRRL 15910 (U.S. Patent No. 4,691,009) was cultivated in an aqueous medium (see Table 2) at 37°C, 800 rpm, 100 min., pH 7.2 ± 0.1 (titrants: 10% NH₄OH, 2 M H₃PO₄). After growth leveled off, the broth was heated to 80°C for 5 min, then cooled to 30°C. SDS-PAGE analysis of the distribution of recombinant Protein A demonstrated that prior to heating, the Protein A was present in the cells and very little was detectable in the supernatant. After heating to 80°C, substantially all of the Protein A was released from the cells was present in the broth supernatant in substantially pure form. Thus, heat inactivation at 80°C results in the release of substantially all of the Protein A into the supernatant and the retention of other proteins within the cells in a manner analogous to Protein G.

-11-

TABLE 2
Fermentation Media Composition

<u>Ingredients</u>	<u>Concentration</u>
Tryptone	30 g/l
Yeast extract	10 g/l
K ₂ HPO ₄	5 g/l
(NH ₄) ₂ SO ₄	3 g/l
MgSO ₄ ·7H ₂ O	0.25 g/l
CaCl ₂ ·2H ₂ O	0.1 g/l
M-44 salts (100 X)	10 ml/l

Having now fully described this invention, it will be understood by those of skill in the art that the same can be performed within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof.

-12-

WHAT IS CLAIMED IS:

1. A method for the isolation of a substantially pure polypeptide expressed by a recombinant host comprising:

(a) cultivating on an aqueous nutrient medium, under recombinant protein producing conditions, a host transformed by a vector containing a DNA sequence encoding a polypeptide, said vector further comprising expression signals which are recognized by said host and which direct the expression of said DNA sequence;

(b) heating said aqueous nutrient medium to 50-100°C for a time not to exceed 1 hour to cause release of substantially pure polypeptide from the host; and

(c) recovering said substantially pure polypeptide from the aqueous nutrient medium.

2. The method of claim 1, wherein said recovering comprises removal of dead cells and insoluble debris from said aqueous nutrient medium.

3. The method of claim 1, wherein said host is a Gram-negative bacterium.

4. The method of claim 1, wherein said host is selected from the group consisting of E. coli, Erwinia sp. and Klebsiella sp.

5. The method of claim 1, wherein said host is E. coli.

6. The method of claim 1, wherein said polypeptide is selected from the group consisting of protein G or a variant thereof having the immunoglobulin-binding properties of

-13-

protein G, or protein A or a variant thereof having the immunoglobulin-binding properties of protein A.

7. The method of claim 1, wherein said vector contains a DNA sequence which encodes protein G or a variant thereof having the immunoglobulin-binding properties of protein G.

8. The method of claim 7, wherein said vector is pGX5204.

9. The method of claim 1, wherein said host is E. coli strain NRRL 15910 which contains a vector which encodes protein A.

10. The method of claim 1, wherein said aqueous nutrient medium is heated to 80°C for about 5 minutes to cause release of substantially pure polypeptide from the host.

11. A method for the isolation of of a substantially pure protein G, or a variant thereof having the immunoglobulin binding properties of protein G, expressed by a recombinant host comprising:

(a) cultivating on an aqueous nutrient medium, under recombinant protein G producing conditions, a host transformed by a vector containing a DNA sequence encoding protein G, said vector further comprising expression signals which are recognized by said host and which direct the expression of said DNA sequence;

(b) heating said aqueous nutrient medium to 50-100°C for a time not to exceed 1 hour to cause release of substantially pure protein G from the host; and

(c) recovering said substantially pure protein G from the aqueous nutrient medium.

-14-

12. The method of claim 9, wherein said host is E. coli.

13. The method of claim 9, wherein said vector is pGX5204.

14. A method for the isolation of a substantially pure protein G variant, having the immunoglobulin binding properties of protein G, expressed by recombinant E. coli which comprises:

(a) cultivating on an aqueous nutrient medium, under recombinant protein G producing conditions, E. coli transformed by the vector pGX5204;

(b) heating the aqueous nutrient medium to 50-100°C for a time not to exceed 1 hour to cause release of substantially pure protein G from said E. coli; and

(c) recovering said substantially pure protein G variant from the aqueous nutrient medium.

15. A method for the isolation of a substantially pure protein G variant having the immunoglobulin-binding binding properties of protein G, expressed from recombinant E. coli which comprises:

(a) cultivating on an aqueous nutrient medium, under recombinant protein G producing conditions, E. coli transformed by the vector pGX5204;

(b) heating the aqueous nutrient medium to about 80°C for about 5 minutes to cause release of said substantially pure protein G; variant and

(c) recovering said substantially pure protein G variant from the aqueous nutrient medium.

1/1

FIG. 1

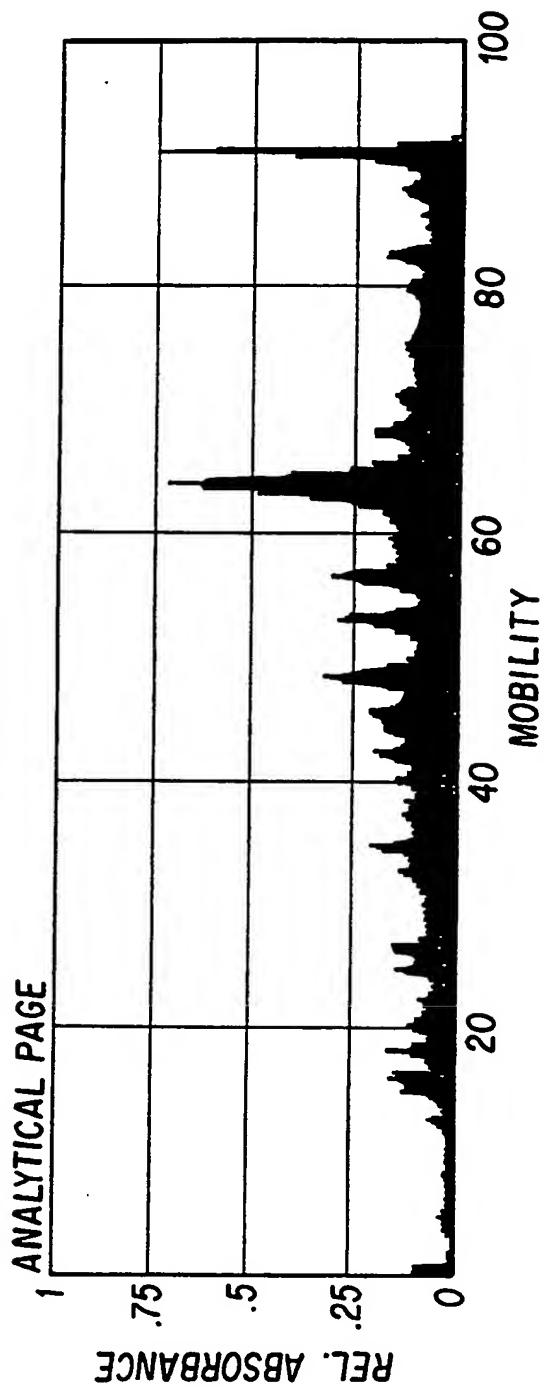
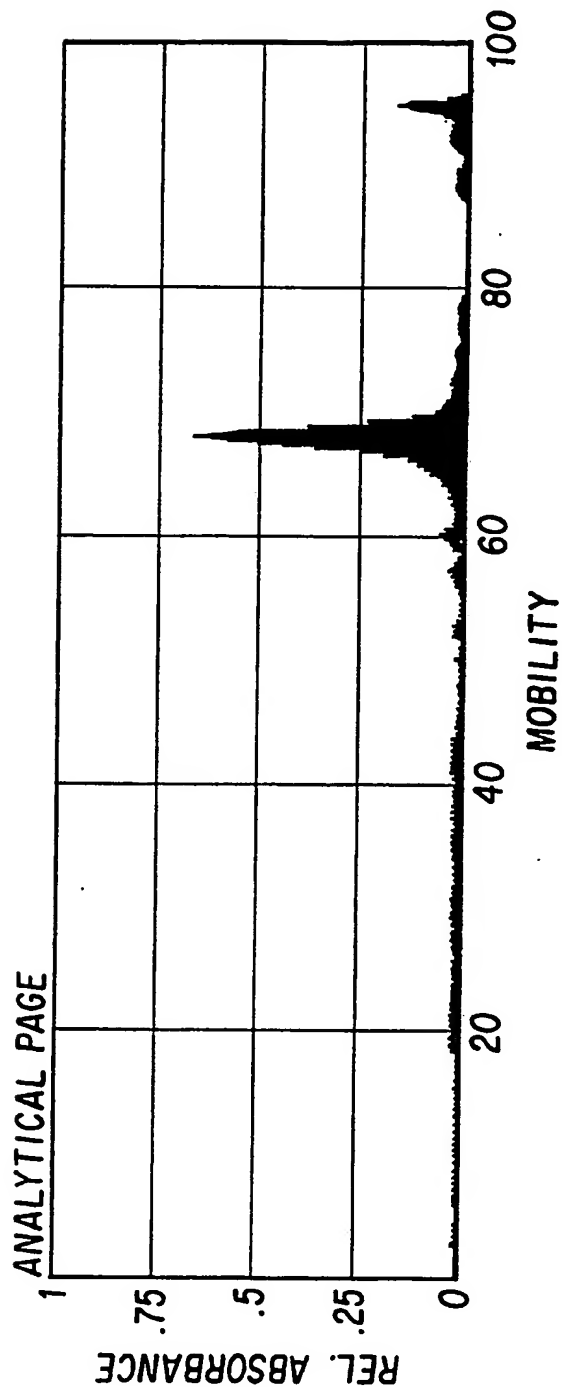


FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/01917**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (4): C12P 21/00, C07K 1/14, C07K 3/02, C07K 3/12, C07K 3/28 U.S. CL.: 435/68 935/60 935/61		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	435/68, 70, 712.1, 172.3, 183, 184, 272 530/419 935/59 , 60, 61, 111	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
Computerized databases: Chemical Abstracts Service (CAS) 1962-1989 and BIOSIS Previews 1969-1988: Keywords: Protein A, <u>protein G, immunoglobulin binding; see attachment.</u>		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Biochimica et Biophysica ACTA, vol. 38, issued 1960, A. Garen and C. Levinthal, A fine structure genetic and chemical study of the enzyme alkaline phosphatase of <u>E. coli</u> , pages 470-483. See especially page 473 last paragraph, continued on 474.	1-15
Y	US, A, 3,585,179, Samejima et al., June 1971. See especially the Abstract of the Disclosure and column 2, lines 65 to 72.	1-15
A	Nucleic Acids Research, Vol. 14, Number 18, issued 1986, Abrahamsen et al., Secretion of heterologous gene products to the culture medium of <u>E. coli</u> , pages 7487-7501.	1-15
A	Scopes, Robert K., "Protein Purification," 1982, Springer-Verlag, New York, pages 61-63.	1-15
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 19 September 1989		Date of Mailing of this International Search Report <div style="font-size: 1.5em; font-weight: bold;">12 OCT 1989</div>
International Searching Authority ISA/US		Signature of Authorized Officer LARRY S. MILLSTEIN, Ph.D.

PCT/US89/01917

Attachment to PCT/ISA/210
Part II. Fields Searched

Keywords: E. coli, klebsiella, Erwinia; heat, thermal, temperature, °C; protein, polypeptide; enzyme; purify, isolate, recover, prepare; precipitate, supernatant; secret; peptidylglycan, periplasmic, proteoglycan; pasteurize, sterilize; recombinant, clone, sequence, cDNA; thermophilic, thermostable, heat stable.

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